NEW HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF DIFFERENT FIXED DOSAGE COMBINATIONS

SYNOPSIS OF THE THESIS

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DECLARATION

I hereby declare that the dissertation synopsis entitled **"Development and validation of HPLC method of different fixed dose combinations"** to be submitted for the partial fulfillment of the Degree of Doctor of Philosophy is my original work and the dissertation has not formed the basis for the award of any degree, diploma, associateship or fellowship of similar other titles. It has not been submitted to any other University or Institution for the award of any degree or diploma.

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1. INTRODUCTION

Chromatography is probably the most powerful analytical technique available to the modern chemist. Its power arises from its capacity to determine quantitatively many individual components present in mixture by single analytical procedure [1]. High-performance liquid chromatography (HPLC) is a chromatographic technique that can separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of the mixture [2]. Reversed phase chromatography has found both analytical and preparative applications in the area of biochemical separation and purification. Molecules that possess some degree of hydrophobic character, such as proteins, peptides and nucleic acids, can be separated by reversed phase chromatography with excellent recovery and resolution [3]. Now a day reversed-phase chromatography is the most commonly used separation technique in HPLC due to its broad application range. It is estimated that over 65% (possibly up to 90%) of all HPLC separations are carried out in the reversed phase mode. The reasons for this include the simplicity, versatility, and scope of the reversed-phase method as it is able to handle compounds of a diverse polarity and molecular mass [4].

Types of HPLC methods

1. Reverse Phase HPLC Reversed phase chromatography has found both analytical and preparative applications in the area of biochemical separation and purification. Molecules that possess some degree of hydrophobic character can be separated by reversed phase chromatography with excellent recovery and resolution [3]. Uses water-organic as mobile phase, columns may be C18 (ODS), C8, phenyl, Trimethyl Silane (TMS), cyano as a stationary phase. It is first choice for most samples especially neutral or non ionized compounds, that dissolve in water organic mixtures.

2. Normal Phase HPLC In this the mixtures of organic solvents for mobile phase and columns i.e. cyano, diol and amino silica can be used as stationary phase. It is first choice for mixtures of isomers and for preparative scale HPLC and second choice for lipophilic samples that cannot dissolve well in water-organic mixtures [5]. The type of the stationary phases, mobile phases, and retention order of the analytes in each of the HPLC modes represents in figure 1.

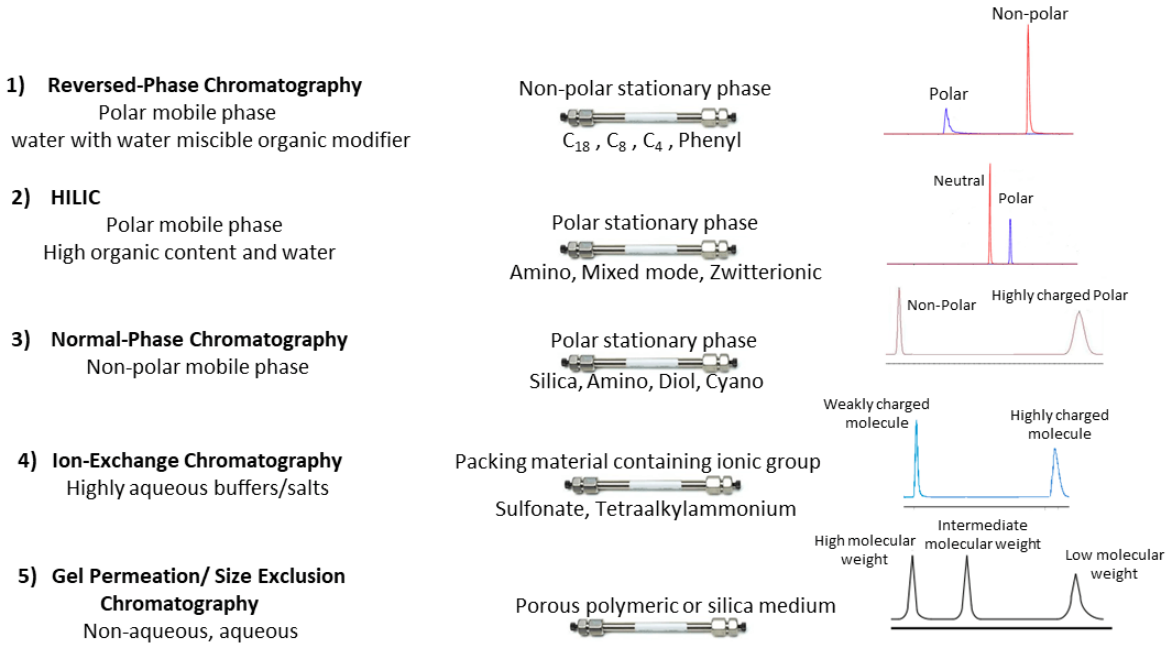


Figure 1: The type of the stationary phases, mobile phases, and retention order of the analytes in each of the HPLC modes.

The HPLC mode selection approach according to physicochemical properties of the target analytes, including molecular weight and solubility represented in table 1.

	Solubility	Type of Solvent	HPLC Mode
Molecular Weight < 2000	Organic	Non-Polar	Normal Phase
		Water Miscible	Reversed Phase
			HILIC (for weak retention in RP)
		THF	Gel Permeation (small molecules)
	Aqueous	Non-ionic	Reversed Phase
			HILIC (for weak retention in RP)
		Ionic	Ion Exchange
			Reversed Phase with Ionization Control
			Reversed Phase with Ion-Pair Agent

	Solubility	HPLC Mode
Molecular Weight > 2000	Organic	Gel Permeation
	Aqueous	Ion Exchange with Wide-Pore Material
		Size Exclusion Chromatography
		Reversed-Phase with Wide-Pore Material

Table 1: The HPLC mode selection approach according to physicochemical properties of the target analytes

The wide variety of equipment, columns, eluent and operational parameters involved makes high performance liquid chromatography (HPLC) method development seem complex. The process is influenced by the nature of the analytes and generally follows the following steps:

- Step 1 - Selection of the HPLC method and initial system
- Step 2 - Selection of initial conditions
- Step 3 - Selectivity optimization
- Step 4 - System optimization
- Step 5 - Method validation.

A flow diagram of an HPLC system is illustrated in Figure 2.

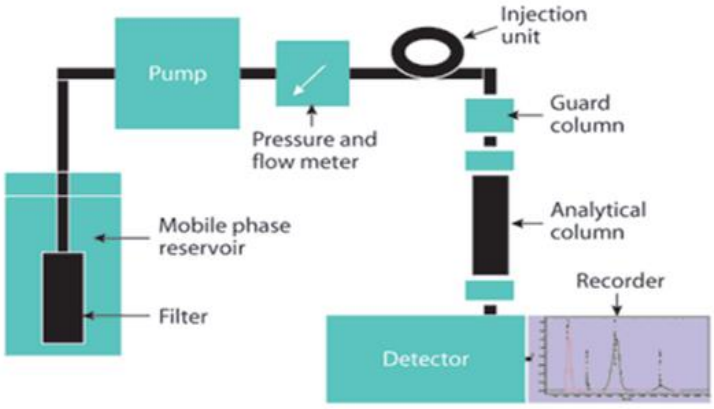


Figure 2: A flow diagram of an HPLC system.

1.1Analytical method validation

There is a general agreement that at least the following validation parameters should be evaluated for quantitative procedures: selectivity, calibration model, stability, accuracy (bias, precision) and limit of quantification. Additional parameters which might have to be evaluated include limit of detection (LOD), recovery, reproducibility and ruggedness (robustness).

a. Linearity
b. Precision
    i. Repeatability
    ii. Intermediate Precision
    iii. Reproducibility
c. Accuracy
d. Specificity
e. Detection Limit
f. Quantitation limit
g. Range
h. Robustness
i. System Suitability
j. Stability

a. Linearity: Linearity assesses the ability of the method to obtain test results that are directly proportional to the concentration of the analyte in the sample. Linearity can be assessed by performing single measurements at several analyte concentrations.

b. Precision: The exactness of an analytical procedure expresses the nearness of agreement (degree of scatter) between a group of measurements obtained from different sampling of a uniform sample underneath the prescribed conditions. Precision may be taken into consideration at 3 levels:

c. Accuracy: Accuracy should be performed at a minimum of three concentration levels. For drug substance, accuracy can be inferred from generating acceptable results for precision, linearity, and specificity. For assay methods, the spiked placebo samples should be prepared in triplicate at 80, 100, and 120%.

d. **Specificity:** For every phase of product development, the analytical method must demonstrate specificity. The method must have the ability to unambiguously assess the analyte of interest while in the presence of all expected components, which may consist of degradants, excipients/sample matrix, and sample blank peaks. The sample blank peaks may be attributed to things such as reagents or filters used during the sample preparation. For identification tests, discrimination of the method should be demonstrated by obtaining positive results for samples containing the analyte and negative results for samples not containing the analyte.

e. **Detection Limit:** The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

   i. **Based on Visual Evaluation:**

   ii. **Based on Visual Evaluation:**

   iii. **Based on the Standard Deviation of the Response and the Slope:**

The detection limit (DL) may be expressed as:

$$DL = \frac{3.3\ \sigma}{S}$$

where σ = the standard deviation of the response

S = the slope of the calibration curve

f. **Quantitation limit:** The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

   i. **Based on Visual Evaluation**:

   ii. **Based on Signal-to-Noise Approach**:

   iii. **Based on the Standard Deviation of the Response and the Slope:**

The detection limit (DL) may be expressed as:

$$DL = \frac{10\ \sigma}{S}$$

where σ = the standard deviation of the response

S = the slope of the calibration curve

g. **Range:** The range of an analytical procedure is the interval between the upper and lower concentration of an analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable precision, accuracy and

linearity. The range is normally expressed in the same units as the test results (for example percentage, parts per million) obtained by the analytical method.

h. Robustness: The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used [10].

Examples of typical variations are:

- Stability of analytical solutions
- Extraction time

In the case of liquid chromatography, examples of typical variations are:

- Influence of variations of pH in a mobile phase
- Influence of variations in mobile phase composition
- Different columns (different lots and/or suppliers)
- Temperature
- Flow rate

i. System Suitability: After a method has been validated, an overall system suitability test should be routinely run to determine if the operating system is performing properly. An acceptable approach is to prepare a solution containing the analyte and a suitable test compound. If the method being used is to control the level of impurities, the minimum resolution between the active component and the most difficult to resolve impurity should be given. The chromatographic system should demonstrate acceptable resolution of the test solution and system precision.

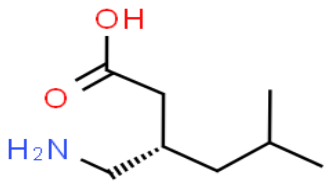
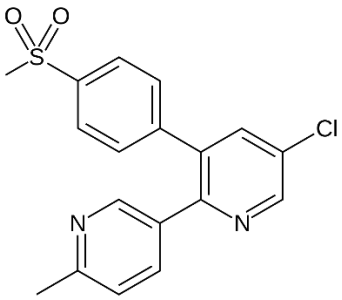
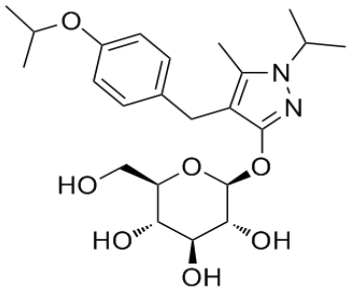
The parameters used in the system suitability tests (SST) report are as follows:

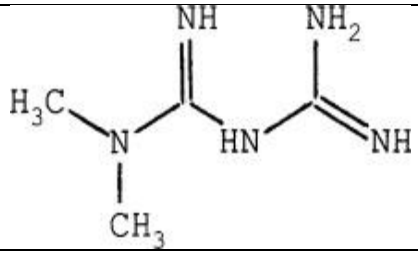
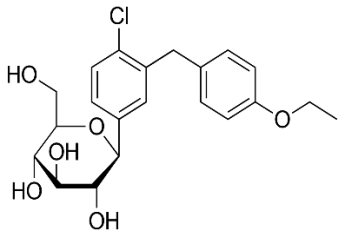
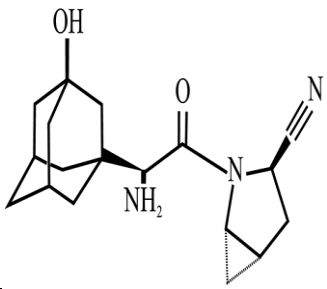
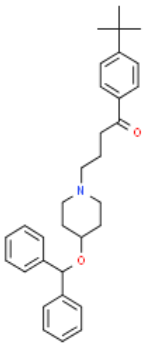
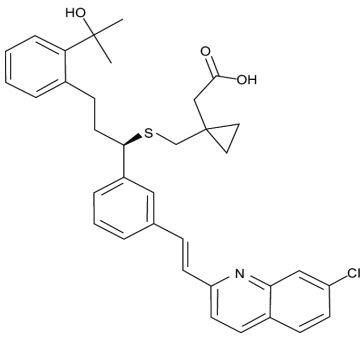
i. **Number of theoretical plates or Efficiency (N):**
ii. **Capacity factor (k'):**
iii. **lnjection repeatability (RSD):**
iv. **Relative retention (α): Tailing factor (T):**

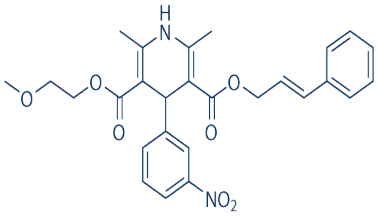
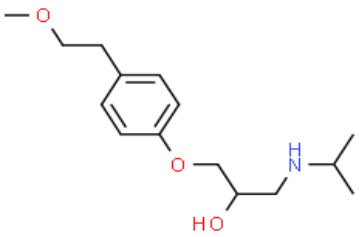
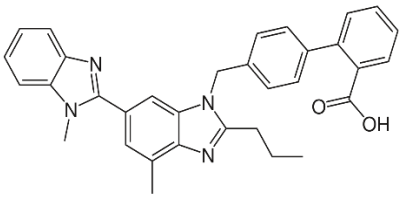
j. Stability: Many analytes readily decompose prior to chromatography investigations, for example during the preparation of the sample solutions, during extraction, clean-up, phase transfer, and during storage of prepared vials.

Under these circumstances, method development should investigate the stability of the analyte. Accuracy test takes care of stability. It is required to mention in the method how long a sample after extraction can be stored before final analysis, based on the duration taken for accuracy test [16].

1.2 List of selected fixed dose combinations

S.No	Combination Name	Chemical structure	Therapeutic Class
1	**Pregabalin + Etoricoxib**	 + 	**Pregabalin: (Newer generation gabapentinoid)** It is used to treat of neuropathic pain conditions including diabetic peripheral neuropathy, postherpetic neuralgia, low back pain with radiculopathy, fibromyalgia and central pain due to spinal cord injury. **Etoricoxib: [Selective inhibitor of cyclooxygenase-2 (COX-2)]** It is an enzyme involved in pain and inflammation.
2	**Remogliflozin + Metformin**	 +	**Remogliflozin:** It is drug of the gliflozin class for the treatment of non-alcoholic steatohepatitis ("NASH") and type 2 diabetes. **Metformin**: is an antihyperglycemic agent of the biguanide

			class, used for the management of type II diabetes.
3	**Dapagliflozin + Saxagliptin**	 + 	**Dapagliflozin**: **(SGLT2 Inhibitors)** reduce blood sugar levels in type 2 diabetes. **Saxagliptin:(dipeptidyl peptidase-4 {DPP-4)}inhibitor)** It is antidiabetic for the treatment of type 2 diabetes.
4	**Ebastine + Montelukast**	 + 	**Ebastine**: **(second-generation histamine H1 receptor antagonist)** It used to attenuate allergic inflammation. **Montelukast**. **(oral leukotriene receptor antagonist)** It is used for the treatment of asthma and seasonal allergic rhinitis (hay fever)

5	**Clinidipine + Metoprolol + Telmisartan**	 +  + 	**Cilnidipine:** It is a dihydropyridine class of calcium-channel blocker used to treat hypertension. **Metoprolol (beta blockers):** It slowing down a person's heart rate and lowering their blood pressure. **Telmisartan: (angiotensin receptor blockers)** It is used to treat high blood pressure (hypertension)

2. LITERATURE REVIEW

A. Introduction

Sharma et al (2018) studied that the top objective of any pharmaceutical industry is to produce products of necessary characteristic and quality reliably, in a cost-effective manner. Development of a method is essential for discovery, development, and evaluation of medicines in the pharmaceutical formulation. The main aim of this review article was to check the development and validation of the procedure employed for the medication from the starting of the formulation to the complete commercial batch of product. At the point when an analytical technique is applied to produce outcomes for the quality of medicine associated samples, it is necessary that the outcomes are reliable. In the pharma industry, validation policy is documented for how to perform validation, types of validation and validation policy are complied with the necessities of good manufacturing practice (GMP) regulations. Validation is very important for the effective running of the pharmaceutical firms. At every stage from raw material to the finished, stability, everywhere validation was performed. The method was developed properly, and validation parameters are explained in terms of accuracy, specificity, precision, limit of detection (LOD), limit of quantitation (LOQ), ruggedness, robustness, and system suitability testing with the example of certain drugs. All validation parameters are used in the routine and stability analysis.

Chatwal and Anand (2016) studied that High-performance liquid chromatography (HPLC) is a chromatographic a technique that can separate a mixture of compounds and also used to identify, quantify and purify the individual components of the mixture in biochemistry and analytical chemistry. RP- chromatography has found both analytical and preparative applications in the area of biochemical separation and purification of proteins, peptides and nucleic acids molecules, which have some degree of hydrophobic characters.

Kumar and Kumar (2012) reviewed that the HPLC is a simple, versatile technique where analytes are separated by passage through a column packed

with micrometer-sized particles. It is also able to handle compounds of a diverse polarity and molecular mass. RP-HPLC has found both analytical and preparative applications in the area of biochemical separation & purification of proteins, peptides and nucleic acids with excellent recovery and resolution. The importance of RP-HPLC in analytical method development and their strategies along with brief knowledge of critical chromatographic parameters need to be optimized for an efficient method development.

Synder et al (2012) demonstrated in their practical experiments based on HPLC method development to explore the knowledge of nature of sample, separation goal sample pretreatment and detection, Selection of HPLC methods, improving the separation develop and design the novel validated HPLC methods.

Tiwari and Tiwari (2010) reviewed that the development of sound bioanalytical method(s) is of paramount importance during the process of drug discovery and development, culminating in a marketing approval. The objective of this paper is to review the sample preparation of drug in biological matrix and to provide practical approaches for determining selectivity, specificity, limit of detection, lower limit of quantitation, linearity, range, accuracy, precision, recovery, stability, ruggedness, and robustness of liquid chromatographic methods to support pharmacokinetic (PK), toxicokinetic, bioavailability, and bioequivalence studies. Bioanalysis, employed for the quantitative determination of drugs and their metabolites in biological fluids, plays a significant role in the evaluation and interpretation of bioequivalence, PK, and toxicokinetic studies. Selective and sensitive analytical methods for quantitative evaluation of drugs and their metabolites are critical for the successful conduct of pre-clinical and/or biopharmaceutics and clinical pharmacologystudies.

Ahuja (2007) reviewed that High-pressure, or high-performance, liquid chromatography (both are abbreviated as HPLC or simply LC; see references 1-2 as to proper usage of these terms) is predominantly used in the pharmaceutical industry for evaluations of a large variety of samples. It is the method of choice for checking purity of new chemical entities, monitoring changes in synthetic

procedures or scale up, evaluating new formulations, and carrying out quality control/assurance of the final drug product.

Lindholm (2004) developed and validated of HPLC methods for analysis of biotechnological synthesis & it is a powerful separation method for the purification of pure compounds from more or less complex sample mixtures.

Scott (2003) studied that Chromatography is the most powerful and versatile technique available to the modern analyst for chemical analysis & also used in preparative purposes. In a single step process it can separate a mixture into its individual components and provide a quantitative estimate of each constituent. Samples may be gaseous, liquid or solid in nature and can range in complexity from a simple blend of two entantiomers to a multi component mixture containing differing chemical species. Furthermore, the analysis is very costly and complex as compare to thin layer chromatography.

B. Pregabalin and Etoricoxib

Vyas et al (2019) developed and validated a simple, economic, selective, precise, accurate RP-HPLC (Reversed phase–HPLC) method for analysis of Amitriptyline Hydrochloride (AMI) and Pregabalin (PGB) in the formulation.
Reversed-phase chromatography was carried out on a C18 column with buffer (potassium dihydrogen phosphate) pH 4.0 and acetonitrile, (40:60 %v/v), as mobile phase at a flow rate of 1 mL/min. The detection was performed at 230 nm (nanometer). The results revealed sharp peaks at 230 nm for PGB and AMI at retention time of 4.4 and 9.6 min, respectively. The detection limits were found to be 3.00 µg/mL and 0.59 µg/mL and quantification limits were found to be 9.11 µg/mL and 1.79µg/ml for Pregabalin and amitriptyline hydrochloride, respectively. The method was validated for accuracy, precision, reproducibility, specificity, robustness and detection and quantification limits, in accordance with ICH (international council of hormonization) guidelines. Thus, the developed RP-HPLC method was found to be suitable for the determination of both the drugs as well as stability samples of tablets containing various excipients.

Parameswari and Arunamma (2018) fabricated a new simple, sensitive, precise, accurate and stability indicating RP-HPLC method for the simultaneous determination of epalrestat and pregabalin in combined tablet dosage form. The chromatogram was achieved with STD recovery column 250 × 4.6 mm, 5µ and mobile phase containing 0.1% ortho phosphoric acid buffer and acetonitrile taken in the ratio of 45: 55 was pumped through column at a flow rate of 1 ml/min. Temperature was maintained at 25 °C. The effluents were monitored at 244 nm by using PDA detector. The retention time of epalrestat and pregabalin were found to be 2.407 min and 3.272 min. The validation of the method was done according to the ICH guidelines for different analytical parameters. The method was found to be linear in the range of 37.5 - 225 µg and 18.75 - 112.5 µg/ml for epalrestat and pregabalin respectively. The assay of marketed formulation was determined and find with 99.22% and 99.07% w/v for epalrestat and pregabalin respectively. The study suggested that this method can be applied successfully for simultaneous estimation of both epalrestat and pregabalin in bulk and combined tablet formulation.

Potluri et al (2017) performed a simple and precise stability indicating RP-HPLC method for simultaneous analysis of drug nortriptyline and pregabalin using BDS (250mm x 4.6 mm, 5m) C18 column at 210 nm of UV detection. Perchloric acid (0.1%) and acetonitrile in the ratio of 55:45 was used as the mobile phase with a flowrate of 1.0 ml/min and linearity response was established over the concentration range of 5-30 µg/ml for nortriptyline and 37.5-225 µg/ml for pregabalin. The active pharmaceutical ingredients recovered for nortriptyline and pregabalin were in the range of 100.60-101.65% and 100.59-101.74% respectively. The methodwas validated and was found to be stability indicating and can be successfully utilized for the quantitative analysis of pharmaceutical tablet dosage formulationscontaining nortriptyline and pregabalin.

Alzweiri et al (2018) reported a reversed phase high performance liquid chromatography (RP-HPLC) was used to develop and validate simultaneous determination for sodium saccharin, vanillin, methyl paraben, etoricoxib, and butyl paraben, in prepared oral suspensions of etoricoxib. Reversed phase

column of octadecylsilane (ODS) C18 with isocratic mobile phase containing methanol, and phosphate buffer of pH 6 in a ratio of 70:30 (v/v). Celecoxib was used as an internal standard at a detection wavelength of 215 nm. The method separated the analytes in a total running time less than 13 min. Linearity was obtained in the calibration curve for all analytes with a R2 value of > 0.999. Furthermore, beta-cyclodextrin (β-CD) and 2-hydroxypropyl-_-cyclodextrin (HP-β-CD) were added, either alone or combined, to prevent the crystal formation, and any unpleasant taste of etoricoxib in oral formulations. After testing both HP-β-CD and β-CD at 3% w/w for each, the results showed that HP-β-CD is more efficient in preventing the crystal formation of etoricoxib in suspensions at room temperature than β-CD.

Gangane et al (2014) developed a simple, precise and specific high performance liquid chromatographic method and validated for the determination of Etoricoxib in bulk and tablet dosage form. The method employed LC-10ATVP Shimadzu Liquid Chromatograph HPLC with Hyper ODS 2 C18 size 4.5x250 mm column as a stationary phase. The solvent system consisted of methanol (HPLC Grade) at flow rate 1 ml/min. The analysis of etoricoxib was carried out at 233 nm. The calibration curve for etoricoxib was linear from 20-55 µg/ml. The interday and intraday precision was found to be within limits. The proposed method has adequate sensitivity, reproducibility and specificity for the determination of etoricoxib in bulk and its tablet dosage forms. LOD and LOQ for etoricoxib were found to be 0.250 and 0.650. Accuracy (recoveries: 99.50-99.73 %) and reproducibility were found to be satisfactory.

C. Remogliflozin and Metformin

Jagadeesh and Annapurna (2019) designed a sensitive, rapid, accurate, and precise stability indicating high-performance liquid chromatography method and validated for the simultaneous estimation of metformin (MET) and ertugliflozin (ERT). The planned chromatographic method was developed using Kromasil C18 column using 0.1 M sodium dihydrogen phosphate methanol (50:50, by volume, pH 4.0) as mobile phase system followed by peak area response measurement at 238 nm. The developed method was validated by means of ICH guidelines about system suitability, linearity, sensitivity, selectivity, accuracy,

precision, robustness, and specificity. In this study, calibration curves of MET and ERT were found linear from 250 to 750 µg/ml and 3.75 to 11.25 µg/ml, respectively. Relative standard deviation was <2.0% and recovery was ~ 100%. Successfully, the developed method was applied for the simultaneous determination of MET and ERT in tablets and to study degradation of MET and ERT in acidic, alkaline, oxidation, thermal, and photolytic degradation conditions. No obstructions from additional common excipients of tablets and degradants were observed. The results recommended method suitability for the analysis of MET and ERT in quality control laboratories.

Khalil et al (2018) investigated the first reversed phase high performance liquid chromatographic method for simultaneous determination of Empagliflozin, Canagliflozin, Dapagliflozin and Metformin and validated to be a simple, sensitive, rapid, specific, precise, and accurate method.Chromatographic separation was achieved on C18 column (250×4.6 mm-5µm p.s) Inertsil® ODSthrough isocratic elution using acetonitrile and 0.05 M potassium dihydrogen phosphate buffer PH4 in a ratio [65:35, v/v] as a mobile phase at flow rate of 1ml/min. UV detection was operated at212 nm and injection volum was 10 µl. Linearity range for Canagliflozin, Dapagliflozin,Empagliflozin and Metformin was 7.5-225, 5-150, 6.5-187.5 and 10-1000 µg/ml, respectively. The proposed method showed good linearity, accuracy, precision and was successfully applied fordetermination of the four drugs in laboratory prepared mixtures and in the seven pharmaceuticaldosage forms and so it is suitable for quality control of them.

Swamy et al (2018) studied a new validated RP-HPLC method for estimation of metformin and canagliflozin in bulk and pharmaceutical dosage form. To optimize the mobile phase, various combinations of organic solvents were used on Primesil C18 250 column. The mobile phase containing a mixture of methanol and phosphate buffer in the ratio of 70:30% v/v was selected at a flow rate of 1.0ml/min for developing the method and the peak with a good shape and resolution were found resulting in short retention time baseline stability and minimum noise. The retention time for metformin and canagliflozin was found to be 2.413 and 3.47 min respectively, quantitative linearity was obeyed in the

concentration range of 50-250 µg/mL for metformin and 5-25 µg/mL for canagliflozin. The limit of detection and limit of quantitation were found to be 2.1µg/ml and 0.0372µg/ml (metformin), 4.13µg/ml and 0.112µg/ml (canagliflozin) respectively, which indicates the sensitivity of the method. The high percentage recovery indicates that the proposed method is highly accurate no interfering peaks were found in the chromatogram indicating that excipients used in tablet formulation didn't interfere with the estimation of the drug by the proposed HPLC method.

Deepan et al (2017) carried out method development studies using HPLC for the simultaneous determination of metformin hydrochloride (MET) and dapagliflozin (DAP) in pharmaceutical dosage forms. The separation was achieved on an Inspire (4.6 x 150mm, 5µm) 5 micro column with isocratic flow. The mobile phase at a flow rate of 1.0mL/smin consisted ofAcetonitrile and 0.1M orthophosphoricacid buffer (70:30, v/v). The UV detection was carried out at 260nm. The retention times for MET and DAP were 2.097min and 3.691min, respectively. Parameters such as linearity, precision, accuracy, specificity andruggedness are studied as reported in the International Conference on Harmonization guidelines. A linear response was observed over the concentration range of 5-25µg/ mL for DAP and 500-2500µg/ mL for MET respectively. Limit of detection and limit of quantification for DAP were 2.98 and 9.95µg/mL and for MET were 3.05µg/mL and 10.07µg/mL respectively. Individual drugs (MET and DAP) were exposed to thermal, photolytic, hydrolytic and oxidative stress conditions. The resultant stressed samples were analyzed by the proposed method. The method gave high resolution among the degradation products and the analytes. The analysis concluded that the method was selective for simultaneous estimation of metformin and dapagliflozin which will help to improve quality control and contribute to stability studies of pharmaceutical tablets containing these drugs.

D. Dapagliflozin and Saxagliptin

Gundala et al (2019) studied a simple, sensitive, precise, accurate and robust high performance liquid chromatographic method for simultaneous estimation of saxagliptin (SAXA) and dapagliflozin (DAPA) in pharmaceutical

formulation. Risk assessment was performed to identify the critical method parameters. Three independent factors; mobile phase composition, flow rate and column temperature were used to design mathematical models. Central composite design (CCD) was used to study the response surface methodology and to study in depth the effects of these independent factors. Desirability function was used to simultaneously optimize the retention time and resolution of SAXA and DAPA. The optimized and predicted data from contour diagram consisted of acetonitrile and ortho phosphoric acid (0.1%) in the ratio of 50:50 respectively, at a flow rate of 0.98 ml/min and column temperature 31.4 °C. Using these optimum conditions baseline separation of both drugs with good resolution and run time of less than 6 min were achieved. The optimized assay conditions were validated according to ICH guidelines. Hence, the results clearly showed that Quality by design approach could be successfully applied to optimize RP-HPLC method for simultaneous estimation of SAXA and DAPA.

Donepudi and Achanta (2019) developed a simple, rapid, sensitive, and validated isocratic reversed phase-high performance liquidchromatography (RP-HPLC) method for the simultaneous estimation of saxagliptin and dapagliflozin in human plasma using linagliptin as internal standard. The analyte separation was achieved using an Eclipse XDB C18 (150 × 4.6 mm × 5 µm) column with a mobile phase consisting of 0.1% ortho phosphoric acid and acetonitrile (50:50) with pH adjusted to 5.0 at 1 mL/min flow rate. The analyte was detected at 254 nm. The retention time of the internal standard, saxagliptin, and dapagliflozin was 2.746, 5.173, and 7.218 min, respectively. The peaks were found to be free of interference. The method was validated over a dynamic linear range of 0.01 to 0.5 µg/mL and 0.05 to 2 µg/mL for saxagliptin and dapagliflozin, respectively, with a correlation coefficient of 0.998. The analytes were found to be stable in human plasma at -28°C for 37 days. The stability, sensitivity, specificity, and reproducibility of this method make it appropriate for the determination of saxagliptin and dapagliflozin in human plasma.

Padamja et al (2018) carried out development of analytical methods for dapagliflozin and saxagliptin in tablet dosage form. Chromatogram was run through standard BDS C8 column (50 × 4.6 mm, 5m) The Mobile phase

containing potassium dihydrogen phosphate: acetonitrile in the ratio 55:45, pH was adjusted to 3.8 with dilute orthophosphoric acid. The solution was pumped through the column at a flow rate of 1 ml/min. The column temperature was maintained at 30°C. Optimized wavelength selected was 210 nm. Retention time of dapagliflozin and saxagliptin were found to be 2.266 min and 2.805 min. % RSD of the dapagliflozin and saxagliptin were found to be 0.5 and 0.5 respectively. % Recovery was obtained as 98.98% and 98.72% for dapagliflozin and saxagliptin respectively. LOD, LOQ values obtained from regression equations of Dapagliflozin and Saxagliptin were 0.20, 0.60 and 0.26, 0.79 respectively. The method can be applied successfully for simultaneous estimation of both dapagliflozin and saxagliptin in bulk and combined tablet formulation.

Deepan and Dhanaraju (2018) performed and developed a simple, fast, and highly selective RP-HPLC method for the determination of dapagliflozin (DAP) and saxagliptin (SAX) in API and tablet dosage form. The separation was done using RP18 (4.6×150 mm, 5 µm particle size) column with acetonitrile: water (60:40). The isocratic elution mode at a flow rate of 1 mL/min, and the analytes were measured at 248 nm. The retention time for DAP and SAX were about 2.091 and 3.249 min, respectively. Calibration curves were found to be linear in the ranges of 100-500 µg/ml for DAP and 50-250 µg/ml for SAX, with correlation coefficients of 0.9998. The detection and quantification values for DAP was 3.0 and 9.98 µg/ml and SAX was 3.02 and 10 µg/ml respectively.

E. Ebastine and Montelukast

Nalini and Kumar (2020) reviewed analytical methods like electrometric methods, ultraviolet spectroscopy, mass spectroscopy, thin layer chromatography, high performance liquid chromatography, high performance thin layer chromatography and tandem spectroscopy for determination of fexofenadine HCl and montelukast sodium in dosage form and in biological matrices. Complete analytical validation parameters reported are discussed in this review for both analytes. Among various analytical methods, HPLC and UV-visible spectrophotometry were found to be the most extensively used methods by the researchers.

Muralidharan et al (2016) developed and validated high performance liquid chromatographic (HPLC) and ultraviolet (UV) methods for the quantitative estimation of Montelukast (ML) in pharmaceutical dosage form. HPLC was carried out by reverse phase-HPLC technique on a C18 column with a good mobile phase consist of 10 mM ammonium acetate and acetonitrile [(pH 5.5 adjusted with orthophosphoric acid (25:75, v/v)]. UV method was developed with the wavelength at 280 nm. Two methods showed good linearity, reproducibility and precision. Both the methods (UV and HPLC) showed no spectral or chromatographic interferences from the tablet excipients. The developed methods were successfully applied to dosage forms. Validation parameters were carried out such as linearity, precision, accuracy, and specificity. The HPLC LOD and LOQ for montelukast were found to be 150 ng/ml and 500 ng/ml. The UV LOD and LOQ for montelukast were found to be 1 µg/ml and 5 µg/ ml. The developed and validated economical methods could be applicable for analysis of ML and monitoring of the quality of available drugs.

Shrikrishna and Nisharani (2015) developed A RP-HPLC method and validated for simultaneous estimation of montelukast and ebastine in bulk as well as in tablet formulation according to ICH guidelines. The chromatographic separations of drugs were achieved on Younglin (S.K) isocratic system with Lichrocart C18 column (4.6 mm×250 mm, 5µm). The mobile phase consisted by methanol and water (80: 20) at pH 3 adjusted by ortho phosphoric acid. The flow rate was adjusted to 1 ml/min and UV detection was carried out at 268 nm. The retention time for montelukast and ebastine were found to be 6.56 and 2.95 min respectively. The detector was showed linear responses over the concentration range 5 – 25 µg/mL for both drugs by showing a good correlation coefficient of 0.999. This proposed method is highly sensitive, precise and accurate which reduces cost of analysis; hence recommended for routine quality analysis in laboratories.

Patel at al (2014) established a reversed phase high performance liquid chromatographic method for the simultaneous determination of ebastine and

phenylephrine hydrochloride, using a pursuit C18 (300 x 3.9) mm; 10 µm (particle size) column as a stationary phase and a mobile phase composed of methanol: 0.02 M ammonium acetate (60:40 v/v), pH 6.7. The retention times of phenylephrine hydrochloride and ebastine were found to be 5.11 min and 7.09 min, respectively. Linearity was established for ebastine and phenylephrine hydrochloride in the range of 4-34 µg/ml. The percentage recoveries of ebastine and phenylephrine hydrochloride were found to be in the range of 99.34-100.32% and 98.92-102.09%, respectively. Both the drugs were subjected to acid, alkali and neutral hydrolysis, oxidation, dry heat and UV degradation. The degradation studies indicated ebastine and phenylephrine hydrochloride to bemore susceptible to oxidative hydrolysis. This method can be successfully employed for simultaneous quantitative analysis of ebastine and phenylephrine hydrochloride and in bulk drugs and formulations.

Rana et al (2013) reported a rapid and sensitive RP-HPLC method with UV detection (244 nm) for routine analysis of montelukast sodium and ebastine in a pharmaceutical formulation (Ebast-M). Chromatography was performed with mobile phase containing a mixture of methanol:acetonitrile:ammonium acetate (80:10:10, % v/v/v), pH of mobile phase was adjusted 5.5 using glacial acetic acid and flow rate was 1.2 ml/min. The method was validated for linearity, accuracy, robustness and intermediate precision. The linearity was established over the concentration range of 0.01−0.06 mg/ml for both drugs. The correlation coefficients (r2) for ebastine and montelukast were 0.9989 and 0.9955, respectively. Statistical analysis of the data showed that the method was precise, accurate, reproducible and selective for the analysis of ebastine and montelukast drugs. The method was successfully employed for the determination of ebastine and montelukast in commercially available tablet dosage form.

F. Clinidipine, Metoprolol and Telmisartan

Suthakaran et al (2018) estimated metoprolol and telmisartan present in dosage forms using HPLC. Chromatogram was performed on Xbridge C18 (5u,4.6x250mm), with a mobile phase composed of methanol: water adjusted pH 3.5 with (58:42% v/v) at flow rate of 1mL/min. The identification was carried out at 224 nm. The linearity range (60-140%) coefficients (0.999) and %

recovery was found to be (98-102%). Retention time was (2.091) & (5.089). The test samples in all formulations were within in their label claims and this technique is used for analysis.

Kachave et al (2016) established a new high performance liquid chromatography method for the quantitation of cilnidipine and valsartan in pharmaceutical formulations. Determination was performed using an ODS C18, 250mm x 4.6mm, 5µm column, a mobile phase containing Methanol: Water (85:15) pH 3 adjusts with ortho-phosphoric acid in isocratic flow rate 1.0 mLmin-1. The method was validated with respect to linearity, precision, robustness, and accuracy. The calibration graphs ranged from 1-5 µg/mL in cilnidipine and 8-40 µg/mL Valsartan Intra- and inter day relative standard deviation values for the standard solutions were 0.5%, 1.64% and 0.22%, 1.62 %. Robustness of relative standard deviation values was 0.334, 0.101 respectively. Total recoveries of cilnidipine and valsartan from the laboratory prepared mixtures were 98.94 % and 99.04 % respectively.

Hinge et al (2015) studied the development and validation of RP-HPLC methods for simultaneous estimation of cilnidipine and metoprolol succinate in their combined tablet dosage form. In HPLC method, C18G (250 x4.6 mm, 5 µm) column was used as stationary phase and methanol: water in the ratio 80: 20, (v/v) (pH adjusted to3.5 with orthophosphoric acid) as mobile phase was used. The flow rate was 1 ml/min and both drugs were quantified at 231.0 nm. The retention time for cilnidipine and metoprolol succinate was found to be 4.092 ± 0.12min and 2.913 ± 0.18 respectively. The linearity range obtained for RP-HPLC method was 2 -10 µg/ml and 10 -50µg/ml for cilnidipine and metoprolol succinate respectively. The method was validated according to the guidelines of International Conference on Harmonization (ICH) and was successfully employed in the estimation ofcommercial formulations.

Madhukar and Kannappan (2014) developed a simple, rapid, and precise reverse phase high performance liquid chromatographic (RP-HPLC) method for simultaneous analysis of cilnidipine (CLN) and metoprolol succinate (MTS) in a tablet dosageform. This method was performed with a Symmetry C18 (4.6 ×

150mm, 5µm) column with 35:65 (v/v) 20mM potassium dihydrogen orthophosphate buffer :methanol as mobile phase at a flow rate of 1.0 ml/min. UV detection at 225 nm; CLN and MTS were eluted with retention times of 3.516 and 4.870 min, respectively. The method was continued and validated in accordance with ICH guidelines. Validation revealed the method is rapid, specific, accurate, precise, reliable, and reproducible. Calibration curve plots were linear over the concentration ranges 10-50µg/mL for CLN, and 25-125µg/mL for MTS. Limits of detection (LOD) were 0.015 and 0.0375µg/ml and limits of quantification (LOQ)were 0.05 and 0.125µg/mL for CLN and MTS respectively. It was also proved study for degradation kinetics of three drugs. It may be extended for its estimation in plasma and other biological fluids.

Rupareliya and Joshi (2013) developed HPLC method for simultaneous estimation of metoprolol succinate and telmisartan using a mobile phase consisting mixture of Methanol: 10 mM potassium dihydrogen phosphate buffer: 10 mM hexane sulphonic acid (80:10:10,v/v/v) at the flowrate of 1ml/min and detection wavelength at 223.0 nm. HiQSil C18 (250 x 4.6 mm, 5µm) column was used as stationary phase. The retention time for metoprolol succinate and telmisartan were 3.067 min and 5.653 min, respectively. Linearity was observed in the concentration range of 5-80 µg/ml for metoprolol succinate and 5-60 µg/ml fortelmisartan. The coefficient of correlation for metoprolol succinate and telmisartan was found to be 0.9990 and 0.9980 respectively. The results of analysis have been validated statistically and by recovery studies. Both the drugs were subjected to acid, alkali and neutral hydrolysis, oxidation, dry heat and photolytic degradation. The degradation products of telmisartan and metoprolol succinate were well resolved from the pure drugs with significant differences in the retention time values.

3. AIM AND OBJECTIVE

The proposed research work emphases the new analytical methods using HPLC to develop a novel, rapid, precise and accurate method for the simultaneous determination of new fixed dose combinations.

To develop novel and cost effective methods for separation and quantification of the simultaneous estimation of new fixed dosage forms and validate them as per ICH guidelines. This empowers the researchers as well as pharmaceutical industry personnel to apply these methods for the quantitative analysis of drugs.

4. PROPOSED RESEARCH METHODOLOGY

Step 1 - Selection of the HPLC method and initial system. When developing an HPLC method, the first step is always to consult the literature to ascertain whether the separation has been previously performed and if so, under what conditions - this will save time doing unnecessary experimental work. When selecting an HPLC system, it must have a high probability of actually being able to analyse the sample; for example, if the sample includes polar analytes then reverse phase HPLC would offer both adequate retention and resolution, whereas normal phase HPLC would be much less feasible [6]. Consideration must be given to the following:

Sample preparation. Does the sample require dissolution, filtration, extraction, preconcentration or clean up? Is chemical derivatization required to assist detection sensitivity or selectivity?

Types of chromatography. Reverse phase is the choice for the majority of samples, but if acidic or basic analytes are present then reverse phase ion suppression (for weak acids or bases) or reverse phase ion pairing (for strong acids or bases) should be used. The stationary phase should be C_{18} bonded.

Gradient HPLC. This is only a requirement for complex samples with a large number of components (.20–30) because the maximum number of peaks that can be resolved with a given resolution is much higher than in isocratic HPLC.

Gradient HPLC will also give greater sensitivity, particularly for analytes with longer retention times, because of the more constant peak width (for a given peak area, peak height is inversely proportional to peak width).

Column dimensions. For most samples (unless they are very complex), short columns (10–15 cm) are recommended to reduce method development time. Such columns afford shorter retention and equilibration times. A flow rate of 1-1.5 mL/min should be used initially. Packing particle size should be 3 or 5 μm.

Detectors. Consideration must be given to the following:

- Do the analytes have chromophores to enable UV detection?
- Is more selective/sensitive detection required (Table 2)?
- What detection limits are necessary?
- Will the sample require chemical derivatization to enhance detectability and/or improve the chromatography?

Type	Limit of detection ($\mu g/cm^3$)	Response	Flow rate sensitivity	Temperature sensitivity	Gradient elution
UV/Visible absorption	10^{-4}	Selective	No	Low	Yes
Fluorescence	10^{-5}	Selective	No	Low	Yes
IR absorption	10^{-3}	Selective	No	Low	Yes
Refractive index	10^{-2}	Universal	No	$\pm 10^{-4}$ °C	No
Conductometric	10^{-2}	Selective	Yes	± 1 °C	No
Amperometric	10^{-5}	Selective	Yes	± 1 °C	_
Mass spectroscopy	10^{-5}	Universal	No	None	Yes

Table 2 : HPLC Detector Comparison

- ***UV wavelength.*** For the greatest sensitivity λ_{max} should be used, which detects all sample components that contain chromophores. UV wavelengths below 200 nm should be avoided because detector noise increases in this region. Higher wavelengths give greater selectivity.
- ***Fluorescence wavelength.*** The excitation wavelength locates the excitation maximum; that is, the wavelength that gives the maximum emission intensity. Selection of the initial system could, therefore, be based on assessment of the nature of sample and analytes together with literature data, experience, expert system software and empirical approaches [7,8].

Step 2 - Selection of initial conditions. This step determines the optimum conditions to adequately retain all analytes; that is, ensures no analyte has a capacity factor of less than 0.5 (poor retention could result in peak overlapping) and no analyte has a capacity factor greater than 10–15 (excessive retention leads to long analysis time and broad peaks with poor detectability). Selection of the following is then required.

Mobile phase solvent strength. The solvent strength is a measure of its ability to pull analytes from the column. It is generally controlled by the concentration of the solvent with the highest strength; for example, in reverse phase HPLC with aqueous mobile phases, the strong solvent would be the organic modifier; in normal phase HPLC, it would be the most polar one. ***Gradient HPLC.*** With samples containing a large number of analytes (.20–30) or with a wide range of analyte retentivities, gradient elution will be necessary to avoid excessive retention.

Determination of initial conditions. The recommended method involves performing two gradient runs differing only in the run time. A binary system based on either acetonitrile/water (or aqueous buffer) or methanol/water (or aqueous buffer) should be used [8].

Step 3 - Selectivity optimization. The aim of this step is to achieve adequate selectivity (peak spacing). The mobile phase and stationary phase compositions need to be taken into account. To minimize the number of trial chromatograms involved, only the parameters that are likely to have a significant effect on selectivity in the optimization must be examined. To select these, the nature of the analytes must be considered. For this, it is useful to categorize analytes into a few basic types (Table 3).

Analyte	**Characteristics**
Neutral	No Significantly acidic or basic functional groups
Weak acid	Has carboxylic acid function or phenolic -OH
Weak base	Aromatic amine
Strong base	Non- aromatic amine

Table 3: The basic types of analyte used in HPLC

Once the analyte types are identified, the relevant optimization parameters may be selected (Table 4). Note that the optimization of mobile phase parameters is always considered first as this is much easier and convenient than stationary phase optimization.

Analyte	**HPLC method**	**Optimize**
Neutral	Reverse Phase	Solvent strength, solvent type
Weak acids and/or weak bases	Ion suppression	pH, solvent strength, solvent type
Strong acids and/or strong bases	Ion pairing	Ion pairing reagent concentration , pH solvent strength, solvent type
Inorganic anions/cations	Ion exchange	Eluting ion concentration

Table 4: The basic types of analyte used in HPLC

Selectivity optimization in gradient HPLC. Initially, gradient conditions should be optimized using a binary system based on either acetonitrile/water (or aqueous buffer) or methanol/water (or aqueous buffer). If there is a serious lack of selectivity, a different organic modifier should be considered.

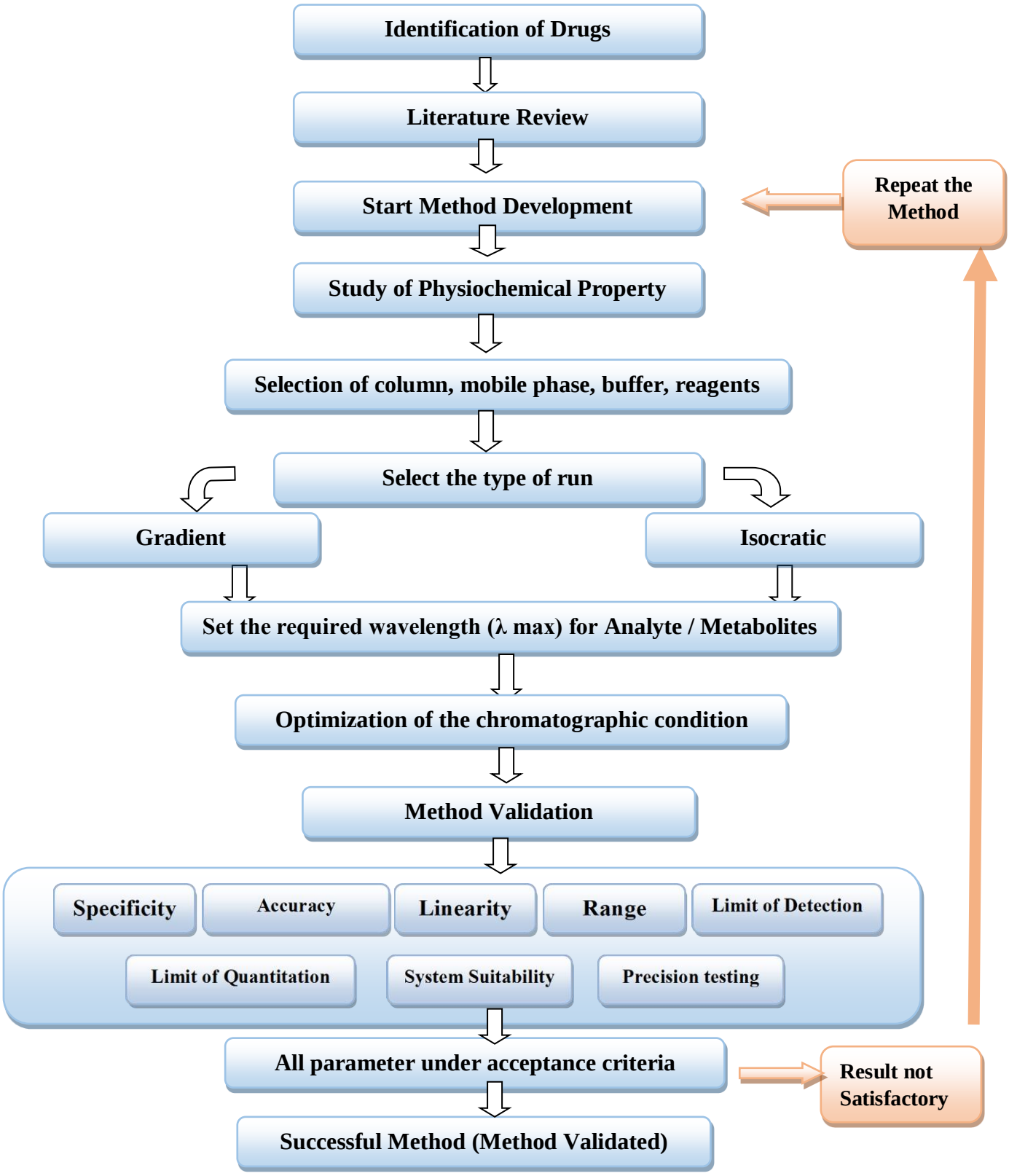
Step 4 - System parameter optimization. This is used to find the desired balance between resolution and analysis time after satisfactory selectivity has been achieved. The parameters involved include column dimensions, column-packing particle size and flow rate. These parameters may be changed without affecting capacity factors or selectivity.

Step 5 - Method validation. Proper validation of analytical methods is important for pharmaceutical analysis when ensurance of the continuing efficacy and safety of each batch manufactured relies solely on the determination of quality. The ability to control this quality is dependent upon the ability of the analytical methods, as applied under well-defined conditions and at an established level of sensitivity, to give a reliable demonstration of all deviation from target criteria.

Analytical method validation is now required by regulatory authorities for marketing authorizations and guidelines have been published. It is important to isolate analytical method validation from the selection and development of the method. Method selection is the first step in establishing an analytical method and consideration must be given to what is to be measured, and with what accuracy and precision.

Analytical methods should be used within good manufacturing practice (GMP) and good laboratory practice (GLP) environments, and must be developed using the protocols set out in the International Conference on Harmonization (ICH) guidelines (Q2A and Q2B). The US Food and Drug Administration (FDA) and US Pharmacopoeia (USP) both refer to ICH guidelines. The most widely applied validation characteristics are accuracy, precision (repeatability and intermediate precision), specificity, detection limit, quantitation limit, linearity, range, robustness and stability of analytical solutions. Method validation must have a written and approved protocol prior to use [9, 10, 11].

5. PLAN OF WORK



Flow Chart of Plan of Work

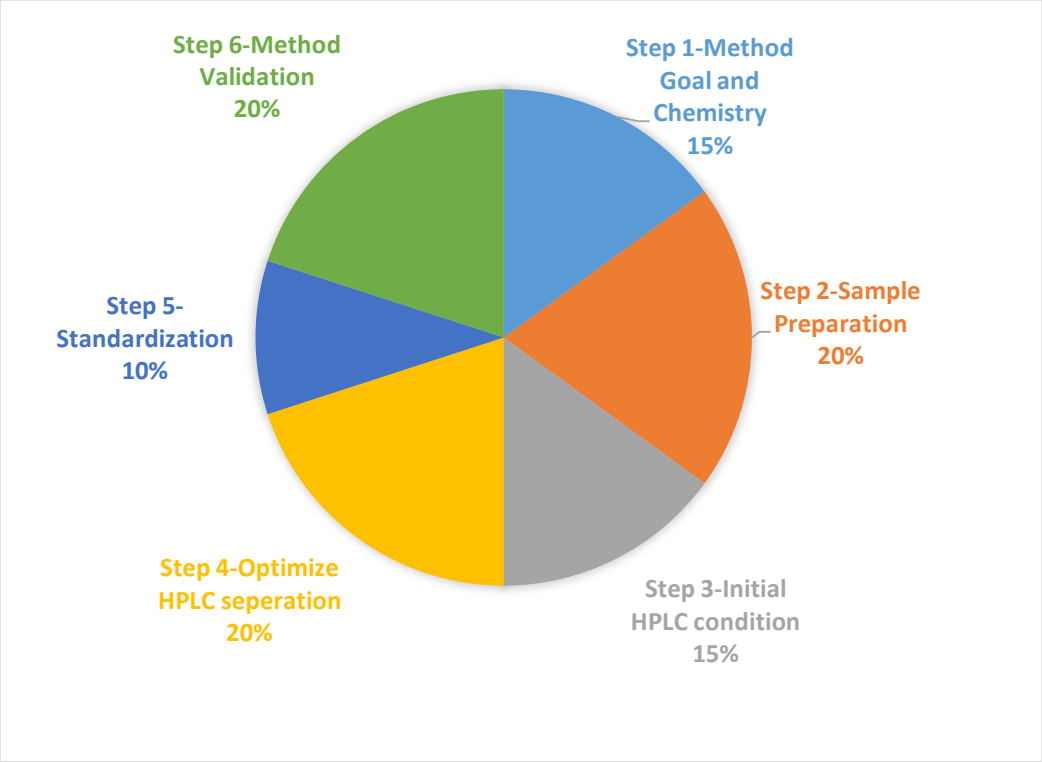

Step 6-Method Validation 20%
Step 1-Method Goal and Chemistry 15%
Step 5- Standardization 10%
Step 2-Sample Preparation 20%
Step 4-Optimize HPLC seperation 20%
Step 3-Initial HPLC condition 15%

Fig: Pie Diagram showing the time that should be spent on different step of method development

6. COMMONLY EQUIPMENT USED

S. NO	Name of equipment's
1	HPLC (Shimadzu LC20AD)
2	Detector SPD-M20A
3	Column ODS-C18
4	UV-Visible Spectroscopy (Labtronics)
5	Ultrasonicator
6	1 mg sensitivity weighing balance
7	Millipore filter 0.45 um Pore Size
8	Filter assembly

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